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[^{3}H]Dofetilide binding in SHSY5Y and HEK293 cells expressing a HERG-like K^+ channel?

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Abstract

The pharmacological characteristics of [^{3}H]dofetilide binding in SHSY5Y, HEK293 and CHO-K1 cells were examined, and in parallel whole cell recordings used to characterise HERG-like K^+ currents. Dofetilide affinity was similar in the human cell lines, SHSY5Y (K_d = 99.6 nM) and HEK293 (K_d = 102.9 nM), but 10 times lower in CHO-K1 cells (K_d = 1200 nM). In contrast, clofilium and E4031 had a similar affinity in all three cell lines, whereas WAY 123,398 had no effect. Electrophysiological studies showed that SHSY5Y cells contained a HERG-like K^+ current blocked by application of dofetilide to either side of the membrane. Block was faster when dofetilide was applied intracellularly. In contrast, HEK293 and CHO-K1 cells contained no such current, despite the presence of a partial cDNA for HERG in the former. That [^{3}H]dofetilide is specific for I_{Kr}/HERG may be questionable, as HEK293 and CHO-K1 cells contain no such functional K^+ current.

Keywords: [^{3}H]dofetilide binding; SHSY5Y cell; HEK293 cell; CHO-K1 cell; HERG (human ether-a-gogo-related gene); RT-PCR (reverse transcriptase-polymerase chain reaction)

1. Introduction

A combination of molecular biological and electrophysiological studies have enhanced our understanding of the roles and potential applications of K^+ channels as putative pharmacological targets (Jan and Jan, 1997; Sanguinetti and Spector, 1997). The K^+ channel encoded by the human ether-a-gogo-related gene (HERG) (Warmke and Ganetzky, 1994) has received a considerable amount of attention. Heterologous expression of HERG in a number of systems including *Xenopus* oocytes and human cell lines has demonstrated that this gene encodes for a K^+ selective channel (Snyders and Chaudhary, 1996; Spector et al., 1996). As with other members of the voltage-gated K^+ channel family, the protein encoded by this gene is predicted to have six transmembrane spanning regions, cytoplasmic N and C termini and a pore forming region between the transmembrane segments S5 and S6 (Jan and Jan, 1997). The main properties of the channel include activation upon depolarisation, small outward currents due to rapid inactivation, which is relieved on hyperpolarisation to exhibit inward rectification, dependence upon extracellular K^+ for maximal conductance and sensitivity to class III antiarrythmics (Bianchi et al., 1998). This current known as I_{Kr} is fundamental to cardiac physiology and mutations in HERG have already been shown to be responsible for chromosome seven linked long QT syndrome (Schonherr et al., 1999). The HERG K^+ channel may also be responsible for the deleterious cardiotoxic side effects that have been associated with the second generation antihistamines and other drugs (Kiehn et al., 1995).

In addition to a key role in the physiology of the heart, it is apparent that HERG K^+ channels are located in other tissues. The cDNA encoding for the channel was first identified from a human hippocampal cDNA library (Warmke and Ganetzky, 1994) and HERG K^+ channels have subsequently been shown to be present in brain, adrenals, thymus and retina (Wymore et al., 1997). In the human cell line SHSY5Y, and in other neuroblastomas, it is postulated that HERG K^+ channels may be involved in setting resting membrane potential (Arcangeli et al., 1995), controlling adhesion-mediated neuritogenesis (Faravelli et

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al., 1996) and in the adult brain may sustain a process of spike frequency adaptation (Chiesa et al., 1997; Schonherr et al., 1999). The identification of mammalian cells lines of neuronal lineage such as SHSY5Y, SK-NBE and F11, which constitutively express HERG-like K^+ channels (Arcangeli et al., 1995; Faravelli et al., 1996; Bianchi et al., 1998; Meyer and Heinemann, 1998), provides an excellent model in which to investigate the biophysical and pharmacological properties of these channels. The characteristic sensitivity of this channel in cardiac tissue to class III antiarrythmics provides a group of compounds with which to characterise the properties of this channel in other tissues. One such compound, dofetilide (UK-68,798) has been radiolabelled and has been used to examine the pharmacology of [^{3}H]dofetilide binding sites (Chadwick et al., 1993; Lynch et al., 1995; Duff et al., 1995, 1997a,b; Fiset et al., 1996; Geonzon et al., 1998).

This paper describes the characterisation of [^{3}H]dofetilide binding sites in the human neuroblastoma cell line SHSY5Y, the human kidney cell line HEK293 and the hamster cell line CHO-K1 and the electrophysiological identification of HERG-like K^+ currents in these cell lines.

2. Materials and methods

2.1. Cell culture

SHSY5Y cells (passage 9–20) were routinely maintained in culture in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 μg/ml) and streptomycin (100 μg/ml) and incubated at 37°C in a humidified atmosphere with 5% CO_2. HEK293 (passage 50–70) and CHO-K1 (passage 19–35) cells were maintained as above in MEM (plus FCS, glutamine and non-essential amino acids) and Ham's F12 (plus FCS and glutamine), respectively.

2.2. [^{3}H]dofetilide binding assay

[^{3}H]dofetilide binding in cell lines was assessed in a physiological sodium buffer by a modification of previous methods (Duff et al., 1995, 1997a; Fiset et al., 1996). Cells were rinsed twice in the sodium incubation buffer (kept at 37°C) (mM: Hepes, 10; NaCl, 130; KCl, 5; $MgCl_2$, 0.8; NaEGTA, 1; glucose, 10; and 0.1% BSA, pH 7.4) and gently scraped from flasks or petri dishes, transferred to 50 ml falcon tubes and centrifuged at $1000 \times g$ for 10 min. Cells were resuspended in 1 ml of sodium incubation buffer and left at 37°C for 30 min prior to counting. Binding assays (see below) were carried out with approximately 10^6 cells per tube; protein content was determined as described previously (Finlayson et al., 1997). Assay buffer or test drug (30 μl) was incubated with 20 μl of [^{3}H]dofetilide (final concentration 10 nM) and 150 μl of cells. The incubation time was 60 min; preliminary experiments confirmed that binding was at equilibrium under these conditions for all three cell lines (data not shown). The binding assay was terminated by filtration onto glass filters (GF/C pre-soaked in wash buffer containing 1% BSA) by use of a Brandel Cell Harvester, followed by three washes (2 ml) with Tris–HCl wash buffer (mM: Tris–HCl, 25; NaCl, 130; KCl, 5.5; $MgCl_2$, 0.8; glucose, 5; $CaCl_2$, 0.05; and 0.01% BSA, pH 7.4). Filter disks were transferred to scintillation vials, allowed to dry prior to the addition of 4 ml of Emulsifier Safe scintillation fluid and radioactivity determined by scintillation counting. For assays without BSA, the procedure was performed exactly as described above, with GF/C filters soaked in 0.25% polyethylenimine (PEI).

2.3. Electrophysiology

Patch-clamp recordings were made at room temperature, using an Axopatch 200 amplifier. All recordings were in the whole-cell mode, using unsylgarded, fire-polished pipettes (Garner glass type 7052) and data recorded directly to a computer using pClamp6. The high potassium extracellular solution used in the bath during recordings, contained (mM): NaCl, 93; KCl, 40; $CaCl_2$, 2; $MgCl_2$, 2; Hepes, 10; glucose, 5; pH 7.4 with NaOH. The pipette solution contained (mM): potassium aspartate, 130; NaCl, 10; $CaCl_2$, 4; $MgCl_2$, 2; EGTA, 10; Hepes, 10; pH 7.3 with KOH. Undifferentiated cells were maintained in culture as described above and used 1–3 days after plating at a density of 250,000 cells per dish (35 mm). Petri dishes were changed every hour and recordings were made in static solutions. Dofetilide was diluted in high potassium solution and added directly to the bath (final concentration 200 nM), or diluted in pipette solution and added to the patch pipette. The HERG inward tail currents were recorded by stepping the voltage to a negative potential from a holding potential of 0 mV. The high potassium extracellular solution maximizes the size of the inward currents when present. At 0 mV, the HERG channels open and inactivate, and the currents subsequently seen at negative potentials are the result of the removal of inactivation. The data was obtained using four different voltage protocols. Protocol 1: used to obtain I/V relationships: 13 voltage steps were applied, at 20-mV intervals, between 80 and −160 mV; steps were 5 s apart, 200 ms in duration and were applied from holding potentials of 0, −40 or −80 mV. Protocol 2: cells were held at 0 mV and stepped to −130 mV for 200 ms; this was repeated every 2 min to obtain a number of control recordings or to follow the effect of a drug application. Protocol 3: cells were held at −60 mV and stepped to 0 mV for 2 s, then to −130 mV for 200 ms, followed by returning to 0 mV for 60 ms and finally back to −60 mV. Protocol 4: nine voltage steps

were applied (20 s apart), each of which contained a 2-s prepulse, followed by a 200 ms step to −130 mV, then returning to the prepulse level for 60 ms and finally back to the holding potential of −60 mV. The prepulses went up from −60 to 100 mV in 20-mV intervals. Data were analysed using the pClamp6 software, and the statistics quoted are for paired *t*-tests.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from SHSY5Y and HEK293 cells (RNeasy, Qiagen, Crawley, UK) and first strand cDNA synthesis performed using 5 μg of total RNA, as per manufacturer's instructions (Amersham Pharmacia Biotech, Bucks, UK). The following sense and antisense primers directed against the HERG sequence (accession number U04270) were used: Sense1; 5′-TCCAGCGGCTGTACTCGGGC-3′ and antisense; 5′-TGGACCAGAAGTGGTCGGAGAACTC-3′ (nucleotides 2171–2190 and 2722–2746, respectively; Arcangeli et al., 1998); Sense2: 5′-GGCTCCATCGAGATCGAGATCCTGCGGGGC-3′ (nucleotides 2530–2559; Bianchi et al., 1998). The first round of PCR was performed using 2 μl of first strand reaction (total volume 15 μl) and the sense1 and antisense primers (0.5 μM final concentrations), by a modification of a previous method (Arcangeli et al., 1998). Briefly, the PCR conditions were as follows: 10 min at 95°C for 1 cycle, 1.5 min at 95°C, 3 min at 50°C and 1.5 min at 72°C for 43 cycles. Five microliters of the first PCR reaction was then used as a template for a subsequent PCR reaction using the sense2 (nested primer) and antisense primer. The PCR conditions for the subsequent reaction were as follows: 94°C for 5 min (1 cycle), 94°C for 30 s, 60°C for 1 min and 72°C for 1 min for 40 cycles, followed by 7 min at 72°C. PCR products were visualized on a 2% agarose gel and bands of the calculated molecular weight were extracted from the gel (Qiagen), verified by restriction digest, subcloned into the PCRII vector (TA Cloning; Invitrogen, Leek, Netherlands) and sequenced using an automated DNA sequencer (Applied Biosystems).

2.5. Data analysis

For [^{3}H]dofetilide binding studies, data were analysed using an iterative, non-linear least square curve fitting program (SigmaPlot, Jandel, USA) to a one-site logistic model; $Y = M \cdot IC_{50}^{P}/(I^P + IC_{50}^{P}) + B$, where P is the Hill coefficient and Y is bound ligand in the presence of inhibitor concentration, I; M and B are specific binding in the absence of inhibitor and non-specific binding, respectively. Estimates of M and B were within 10% of experimentally determined values. If the inhibitor was the unlabelled form of the radioligand, the binding site affinity, K_d and the binding site density, B_{max} were calculated using the equations: $K_d = IC_{50} - [^3H]ligand$ and $B_{max} = (M \cdot IC_{50})/[^3H]ligand$. For other test compounds, K_i values were calculated using the Cheng–Prusoff approximation (Cheng and Prusoff, 1973); $K_i = IC_{50}/(1 + ([^3H]ligand/K_d))$. Statistical analysis was performed using a one- and two-way ANOVA, following demonstration of normality and equality of variance within the data, except where otherwise reported. Significant main effects were further inspected using post-hoc Student–Newman–Keuls.

2.6. Materials

All three cell lines were purchased from the European Collection of Cell Culture (Salisbury, UK). HBSS and cell culture media was from Life Technologies (Paisley, UK) and Tris–HCl was from Fisher Scientific (Loughborough, UK). [^{3}H]dofetilide (80 Ci/mmol) and unlabelled dofetilide (*N*-[4-(2-{2-[4-(methanesulphonamido)phenoxy]-*N*-methylethylamino}ethyl)phenyl]methanesulphonamide) were kindly provided by Pfizer Central Research (Sandwich, UK) and E4031 by the Eisai Pharmaceuticals (Tsukuba, Japan). WAY 123,398 was kindly provided by Wyeth-Ayerst Research (Princeton, NJ, USA) and clofil-

Table 1
Affinity values of various class III antiarrythmic compounds for [^{3}H]dofetilide binding sites in SHSY5Y, HEK293 and CHO-K1 cells

Cell line	Sodium incubation buffer (plus BSA)		Sodium incubation buffer (no BSA)		
	Dofetilide K_d (μM)	Clofilium K_i (μM)	Dofetilide K_d (μM)	Clofilium K_i (μM)	E4031 K_i (μM)
SHSY5Y	0.100 ± 0.032 (n = 5)	1.44 ± 0.83 (n = 3)	0.087 ± 0.010 (n = 4)	0.996 ± 0.578 (n = 4)	4.02 ± 0.78 (n = 4)
(n_H)	1.00 ± 0.06	0.79 ± 0.16	1.19 ± 0.11	0.80 ± 0.13	1.25 ± 0.17
HEK293	0.103 ± 0.020 (n = 8)	0.893 ± 0.170 (n = 4)	0.094 ± 0.016 (n = 6)	0.640 ± 0.122 (n = 4)	3.81 ± 0.73 (n = 5)
(n_H)	0.97 ± 0.07	1.07 ± 0.10	0.94 ± 0.05	1.02 ± 0.06	0.83 ± 0.06
CHO-K1	1.20 ± 0.373 (n = 3)	1.42 (n = 1)	0.844 ± 0.199 (n = 4)	1.14 ± 0.224 (n = 4)	2.67 ± 0.17 (n = 4)
(n_H)	0.98 ± 0.09	1.26	0.89 ± 0.09	0.87 ± 0.11	0.92 ± 0.10

Inhibition of [^{3}H]dofetilide (10 nM) binding in SHSY5Y, HEK293 and CHO-K1 cells in the absence and presence of BSA in the assay buffer. Cells were washed and resuspended in the appropriate buffer and [^{3}H]dofetilide binding performed as described in Materials and methods. K_d/K_i values are mean ± SEM from independent experiments, n_H is the Hill slope and n is the number of determinations.

ium tosylate was from Research Biochemicals (Natick, USA). D-sotalol, bovine serum albumin, PEI and other chemicals were from Sigma (Poole, UK). With the exception of dofetilide (10^{-2} M in DMSO; final concentration 0.1%), drugs were dissolved in H_2O.

3. Results

3.1. Characterisation of [^{3}H]dofetilide binding sites

The affinity of dofetilide, using the method of Duff et al. (1995), which included high levels of BSA in the incubation, wash and filter soaking buffers to maximise specific binding, is similar in the human cell lines, SHSY5Y ($K_d = 99.6 \pm 31.9$ nM; $n = 5$) and HEK293 ($K_d = 102.9 \pm 20.3$ nM; $n = 8$), but significantly lower in the hamster cell line CHO-K1 ($K_d = 1200 \pm 373$ nM; $n = 3$; $F_{(2,13)} = 14.8$, $P < 0.001$) (Table 1). In contrast, the affinity of clofilium was not significantly different in the human cell lines, SHSY5Y and HEK293, with K_i values of 1.44 ± 0.83 ($n = 3$) and 0.89 ± 0.17 μM ($n = 4$), as determined by ANOVA on ranks ($H = 0.125$, $P > 0.05$) (Table 1). In CHO-K1 cells, the affinity of clofilium (1.42 μM, $n = 1$) lay between that of the other two cell lines (Table 1). These data and that obtained with D-sotalol in HEK293 cells ($K_i = 30.4 \pm 4.99$ μM; $n_H = 0.96 \pm 0.07$ ($n = 4$)) demonstrate a rank order of affinity identical to that in previous [^{3}H]dofetilide binding and electrophysiological studies for I_{Kr} (Duff et al., 1995, 1997a; Fiset et al., 1996). The Hill coefficients for all the class III antiarrythmic compounds in each of the three cell lines were close to unity, indicating the presence of a single affinity state or multiple states of equal affinity. Surprisingly, WAY 123,398, another well characterised class III antiarrythmic, had no effect on [^{3}H]dofetilide binding in any of the three cell lines.

3.2. Effect of altering the assay conditions

In previous [^{3}H]dofetilide binding studies (Chadwick et al., 1993; Lynch et al., 1995; Duff et al., 1995), assay buffers contained high levels of BSA in an attempt to maximise the level of specific binding. However, these concentrations of BSA can considerably reduce the speed of filtration and could result in the loss of a low affinity-binding component. In an attempt to address this point, all the BSA was removed from the incubation, filtration and filter soaking buffers; the ionic composition of the incubation and wash buffers were unaltered and GF/C filters were soaked in 0.25% PEI.

Removal of BSA from the assay resulted in no alteration in the level of specific binding for any of the three cell lines (50–60% for SHSY5Y, 75–85% for HEK293

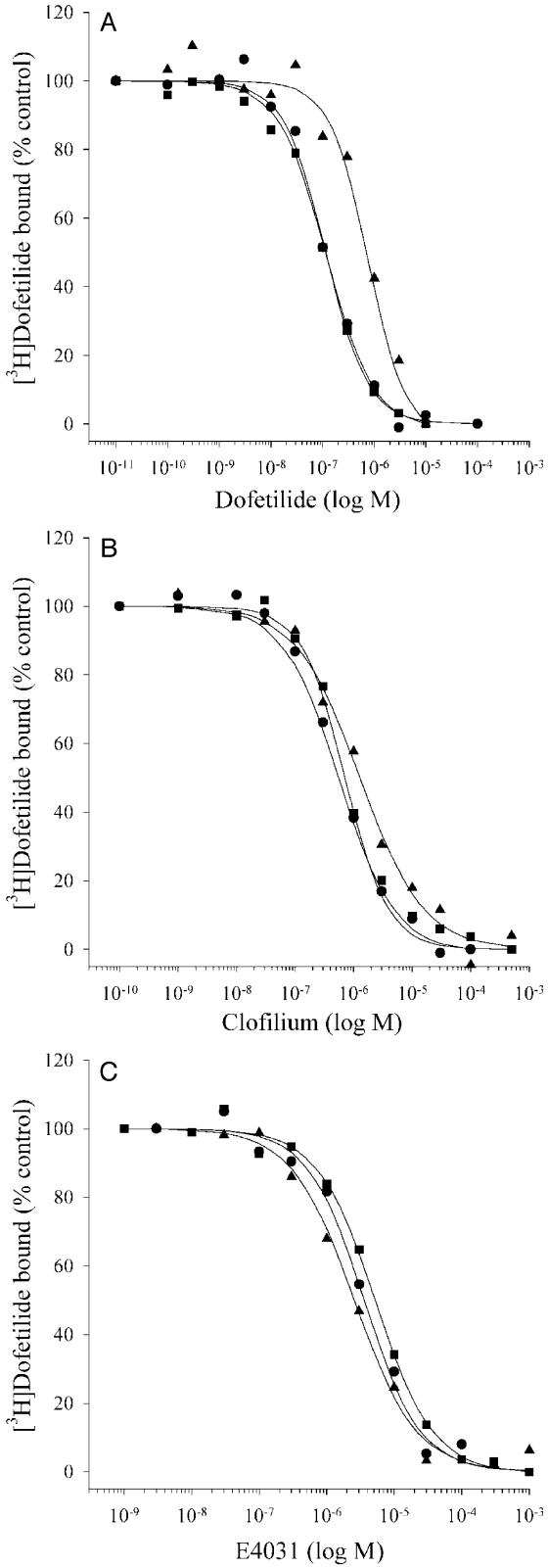

Fig. 1. Inhibition of [^{3}H]dofetilide binding in SHSY5Y (●), HEK293 (■) and CHO-K1 (▲) cells by (A) dofetilide, (B) clofilium and (C) E4031. Cells were washed and resuspended in assay buffer and [^{3}H]dofetilide binding performed as described in Materials and methods. Data shown are representative competition curves from a single experiment; K_d / K_i values were determined from independent experiments as described in Table 1.

and 50–60% for CHO-K1 cells) and dramatically increased the speed of filtration. The affinities of dofetilide ($F_{(1,24)} = 0.09$, $P > 0.05$) and clofilium ($F_{(1,14)} = 0.89$, $P > 0.05$) were not significantly different from those values in the presence of BSA across all three cell lines using a two-way ANOVA (Table 1; Fig. 1A and B). In HEK293 cells, the affinity of D-sotalol was also unaltered by removal of BSA ($K_i = 27.8 \pm 9.7$ μM; $F(1,6) = 0.31$, $P > 0.05$; $n_H = 0.97 \pm 0.05$ ($n = 4$)). The B_{max} values of 1.37 $\pm$ 0.15 ($n = 4$), 5.05 $\pm$ 0.91 ($n = 6$) and 11.2 $\pm$ 2.7 pmol/mg ($n = 4$) for SHSY5Y, HEK293 and CHO-K1, respectively, were significantly different in each cell line ($F_{(2,11)} = 24.7$, $P < 0.05$). In addition, another well characterised class III antiarrythmic E4031 inhibited binding in all three cell lines, with no significant difference in affinity for each cell line ($F_{(2,10)} = 1.27$, $P > 0.05$) (Table 1; Fig. 1C).

3.3. Whole-cell electrophysiology

Whole-cell recordings from undifferentiated SHSY5Y cells identified an endogenous HERG-like K^+ current (Fig. 2A; $n = 7$), similar to that described in previous reports (Arcangeli et al., 1995; Faravelli et al., 1996). When the cell is stepped to negative potentials after holding at 0 mV, the HERG current is seen as a large inward tail current (Fig. 2A); however, no HERG current is seen when the cell is held at −40 mV (Fig. 2B). The current illustrated in Fig. 2A was identified as HERG on the basis of its appearance and kinetics (Arcangeli et al., 1995; Faravelli et al., 1996) and because it was blocked by 0.5 μM WAY 123,398 ($n = 5$; Fig. 2C) and by 10 mM Ba^{2+} (data not shown).

In SHSY5Y cells ($n = 3$), recordings were made using Protocol 2, before and after application of dofetilide (200

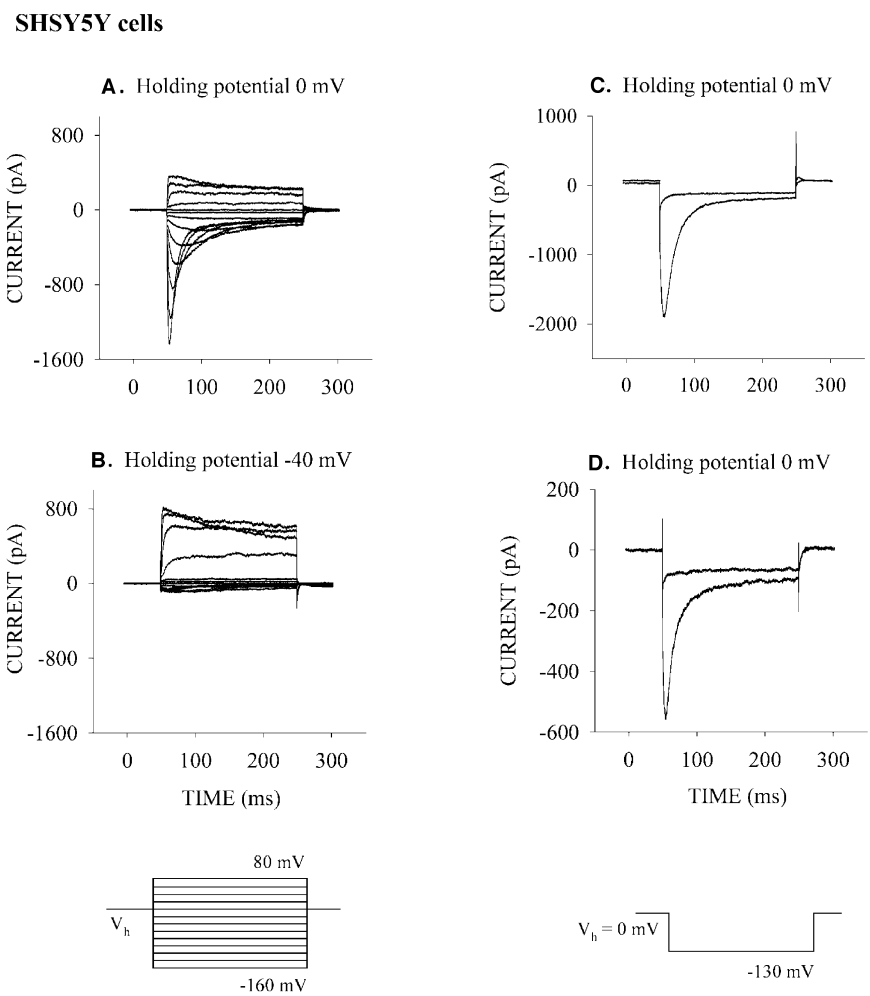

Fig. 2. (A and B) Families of currents obtained from SHSY5Y cells using the protocol shown in the insert with voltage steps applied 5 s apart (protocol 1). Currents activated from a holding potential of 0 mV (A) or −40 mV (B). (A) shows the presence of a large inward HERG-like K^+ current that is not activated in (B). Currents in (C) and (D) were produced by the voltage step shown in the insert (protocol 2). (C) Effect of WAY 123,398 (500 nM) on the HERG-like K^+ current. Lower trace is control and upper trace is 4 min after drug application. (D) Effect of bath applied dofetilide (200 nM) on HERG-like K^+ current. Lower trace is control and upper trace is 11 min after dofetilide application.

nM) to the bathing solution. Dofetilide completely blocked the HERG current, leaving only a small plateau current (Fig. 2D) and caused a significant reduction in peak current (control = -737 ± 107 pA; after dofetilide = -163 ± 43 pA, mean $\pm$ SEM ($n = 3$); $P < 0.05$). Dofetilide did not block the current immediately on application to the bath, with the full effect occurring 11, 13 and 21 min after application and the data quoted "after dofetilide" are for these time points, when the effect was maximal in each cell.

Application of dofetilide (200 nM) via the patch pipette to the inside of the cell demonstrated that block was faster when the drug was applied in this manner and was dependent on the holding potential. In one group of SHSY5Y cells, 0 mV was applied to the patch pipette before, during and after the establishment of whole-cell recording configuration. Protocol 2 was applied and these cells showed no peak in their inward current, i.e. no HERG currents ($n = 3$); dofetilide blocks HERG current immediately when applied to the inside of the cell and when HERG channels have been opened by holding the cell at 0 mV.

In a second group of SHSY5Y cells, -50 mV was applied to the patch pipette before and during the establishment of the whole-cell recording configuration and cells were subsequently held at -60 mV. Protocol 3 (including a prepulse to 0 mV) was used to show HERG currents were unimpaired under these conditions in the presence of 200 nM dofetilide (peak current = -750 ± 286 pA, mean $\pm$ SEM, $n = 3$). However, these currents were blocked as soon as the holding potential was changed to 0 mV (on changing to 0 mV current = -214 ± 41 pA, mean $\pm$ SEM, $n = 3$, currents recorded using Protocol 2). These results confirm that dofetilide cannot block HERG channels while they are held closed at -60 mV. In addition, the channels only become susceptible to block by dofetilide when held at 0 mV for longer than 2 s; the 2 s prepulse to 0 mV in Protocol 3 was not long enough for block to develop.

Recordings were made from HEK293 ($n = 5$) and CHO-K1 ($n = 2$) cells using the conditions described for SHSY5Y cells. I/V plots from holding potentials of 0, -40 and -80 mV were recorded for both cell types and the absence of a HERG-like K^+ current demonstrated. This can be seen clearly when comparing the HERG current recorded from SHSY5Y cells (Fig. 2A) with that of HEK293 and CHO-K1 cells (Fig. 3A and B) at a holding potential 0 mV.

In HEK293 cells ($n = 3$), recordings were made using Protocol 4, before and after application of dofetilide (200 nM) to the bathing solution. The outward currents were measured at their minimum and maximum and results demonstrate that the current present in these cells was not significantly affected by dofetilide (Fig. 4). For voltage steps with a 2-s prepulse to 100 mV, the minimum control outward current was 253 ± 102 pA, and 37 min after dofetilide application, the current was 278 ± 107 pA ($n = 3$, $P > 0.05$), whereas the maximum control outward current was 351 ± 139 pA, and 37 min after dofetilide addition, the current was 487 ± 215 pA ($n = 3$, $P > 0.05$).

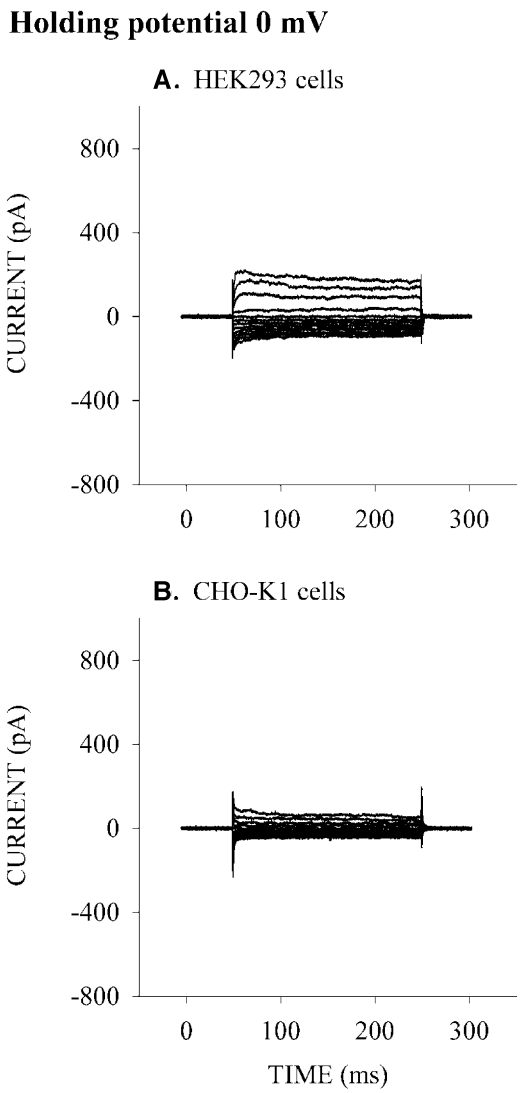

Fig. 3. Families of currents in (A) HEK293 and (B) CHO-K1 cells were recorded from a holding potential of 0 mV using protocol 1. (A) In HEK293 cells, there was no HERG-like K^+ current; however, there was a small unidentified outward current. (B) Recordings from CHO-K1 cells exhibited very little current of any kind.

3.4. Amplification of a HERG channel partial cDNA by RT-PCR

As no functional evidence could be provided for the presence of a HERG-like K^+ channel in HEK293 cells, despite similar [^{3}H]dofetilide binding data to the other human cell line, SHSY5Y, which is known to express HERG (Bianchi et al., 1998), we examined HERG channel expression using RT-PCR. Primers that have been used previously to show the existence of HERG transcripts in SHSY5Y cells (Arcangeli et al., 1998; Bianchi et al., 1998) were used to examine the presence of HERG in both SHSY5Y and HEK293 cells. As the sense1 and antisense

primers (Arcangeli et al., 1998) can produce bands of multiple sizes when aligned against the HERG sequence, this PCR reaction was used as a template for a second PCR reaction using a nested primer (sense2), in conjunction with the antisense primer, to confirm specificity. On an agarose gel, the size of the fragment was as expected (217 bp) in both HEK293 and SHSY5Y cells (Fig. 5, lanes 4 and 5, respectively). In addition, digestion of these PCR products by *Apa*1, resulted in bands of the predicted molecular weights (Fig. 5, lanes 2 and 3, respectively).

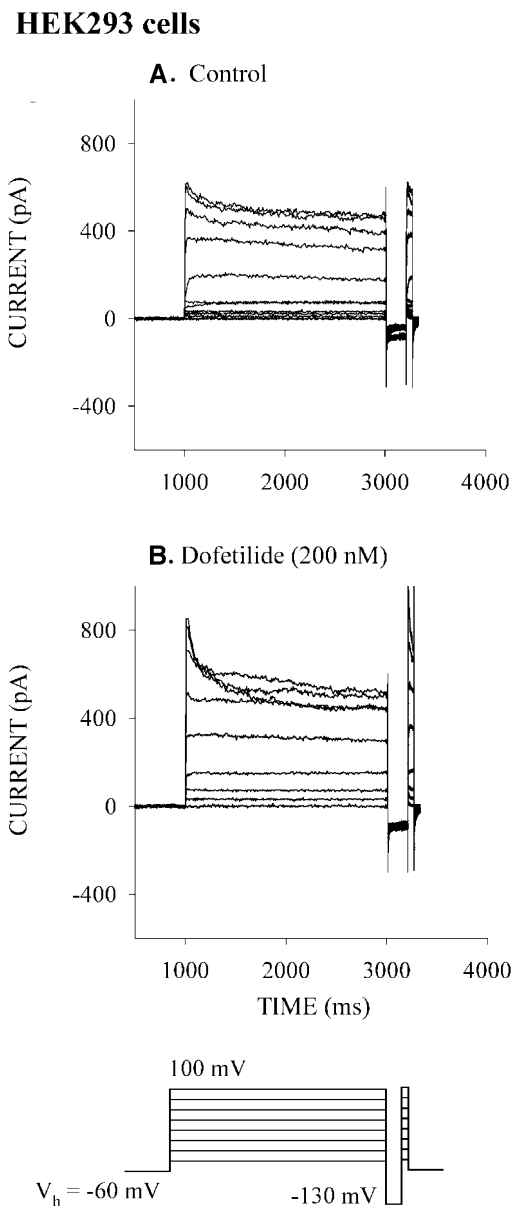

Fig. 4. Effect of dofetilide (200 nM, $n = 3$) on the current present in HEK293 cells generated from a holding potential of -60 mV using the protocol shown in the insert (protocol 4). (A) Control currents and (B) currents 37 min after bath application of dofetilide were not significantly different. For the voltage steps with a prepulse to 100 mV, the minimum outward current was 454 pA in control cells (A) and 487 pA, 37 min after dofetilide (B).

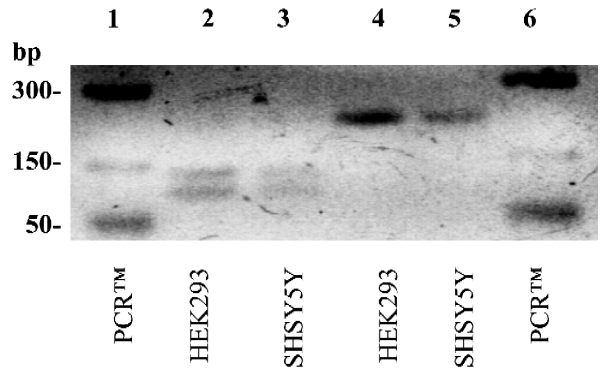

Fig. 5. Agarose gel (2%; ethidium bromide staining) electrophoresis of PCR amplification products from HEK293 and SHSY5Y cells. Lanes 1 and 6, PCR™ markers (Promega, Southampton, UK). Lane 2, *Apa*1 digest of HEK293 PCR product. Lane 3, *Apa*1 digest of SHSY5Y PCR product. Lane 4, uncut 293 PCR product. Lane 5, uncut SHSY5Y PCR product.

The amplified partial cDNAs from HEK293 and SHSY5Y cells were subcloned and sequenced. Both were found to encode for the appropriate region of the HERG sequence (data not shown).

4. Discussion

In this report we describe for the first time, to our knowledge, the characterisation of [^{3}H]dofetilide binding to the two human cell lines SHSY5Y and HEK293. The affinity of dofetilide ($\sim$ 100 nM) in the neuroblastoma cell line SHSY5Y and in the embryonic kidney cell line HEK293 was almost identical and an order of magnitude higher than that observed in the hamster cell line CHO-K1. In contrast, the class III antiarrythmics clofilium and E4031 had a similar affinity in all three cell lines, whereas WAY 123,398 had no effect on [^{3}H]dofetilide binding. Removal of BSA from the assay had no discernible effect on the level of specific binding or on the affinity of the different compounds in any of the three cell lines. Electrophysiological studies identified the presence of a HERG-like K^+ current in SHSY5Y cells, which is in good agreement with previous studies (Arcangeli et al., 1995, 1998; Faravelli et al., 1996; Rosati et al., 1998). However, no such current was identifiable in HEK293 or CHO-K1 cells, despite the similarity in binding data for HEK293 cells and the identification of a partial HERG channel cDNA.

Previously, it has been demonstrated that [^{3}H]dofetilide binds to I_{Kr}, i.e. HERG in both cardiac myocytes and ventricular homogenate from different species (Chadwick et al., 1993; Lynch et al., 1995; Duff et al., 1995). In the present study, we have examined the pharmacology of [^{3}H]dofetilide binding sites in the human neuroblastoma cell line SHSY5Y from which a cDNA encoding for HERG has been identified (Bianchi et al., 1998) and which

is well characterised electrophysiologically (Arcangeli et al., 1995, 1998; Faravelli et al., 1996; Rosati et al., 1998). In SHSY5Y cells, the affinity of dofetilide (~100 nM) in the absence or presence of BSA is in good agreement with that of Chadwick et al. (1993) ($IC_{50} = 100 \pm 30$ nM) who originally characterised [^{3}H]dofetilide binding in guinea pig cardiac myocytes. In contrast to studies in myocytes (Duff et al., 1995, 1997a; Lynch et al., 1995; Fiset et al., 1996), Hill slopes for all compounds were near unity, providing no evidence for more than one affinity site (Table 1). The removal of BSA from the assay resulted in a dramatic increase in the speed of filtration but produced no evidence for an additional low affinity site. However, the affinities of dofetilide, clofilium and E4031 in SHSY5Y cells are, in general, close to the high affinity values reported for each compound in cardiac myocytes and are in excellent agreement with the results of these authors when the data is fitted to a one-site model (Duff et al., 1995, 1997a; Lynch et al., 1995; Fiset et al., 1996).

Dofetilide has been reported to be an open channel blocker of I_{Kr}/HERG at nanomolar concentrations (Snyders and Chaudhary, 1996; Ficker et al., 1998). Our results confirm that in SHSY5Y cells dofetilide is unable to block HERG when the channels are closed. Dofetilide block is more rapid when the drug is added intracellularly, confirming earlier suggestions that dofetilide may access its binding site from inside the cell membrane (Kiehn et al., 1996; Zou et al., 1997; Ficker et al., 1998; Pennington et al., 2000). Our finding that channels need to be held at 0 mV for longer than 2 s before they are susceptible to block by dofetilide suggests something more has to happen to the channel than simply opening before it can be blocked. It is possible that the channels need to be in the inactivated state before dofetilide can bind, as for RP58866 (Wang et al., 1999), or that allosteric changes occur during inactivation (Lees-Miller et al., 2000).

Unlike SHSY5Y cells, we found no evidence for the presence of a HERG-like K^+ current in HEK293 cells, although they do contain a small outward current identified by other authors (Snyders and Chaudhary, 1996; Zhou et al., 1998). In addition, anti-HERG antibodies provided no evidence for the presence of HERG in untransfected HEK293 cells (Zhou et al., 1998). We were therefore somewhat surprised to find [^{3}H]dofetilide binding in HEK293 cells with pharmacological characteristics similar to that described above for SHSY5Y cells (Table 1). As in SHSY5Y cells, Hill slopes for dofetilide, clofilium, E4031 and, in addition, D-sotalol were close to unity. The density of binding sites was higher (~4-fold) and the level of specific binding greater in HEK293 cells than in SHSY5Y cells. So, if there is no functional or biochemical evidence for the existence of HERG in HEK293 cells, what is dofetilide binding to? Although dofetilide was initially thought to be a selective blocker of I_{Kr} (Chadwick et al., 1993), studies indicate that dofetilide and other class III antiarrythmics have affinities for a variety of potassium channels, including the human inward rectifier (hIRK), small-conductance Ca^{2+}-activated channels (SK_{Ca}), the muscarinic acetylcholine receptor-operated channel (IK_{ACh}) and ether-a-gogo-related channels (EAG) (Mclarnon and Wang, 1991; Mori et al., 1995; Kiehn et al., 1995; Geonzon et al., 1998; Lees-Miller et al., 2000). These data indicate that dofetilide could bind to a different K^+ channel in HEK293 cells; however, the small outward current identified in the present study was not blocked by dofetilide. It is therefore possible that [^{3}H]dofetilide binds to a non-functional channel protein or subunit. Indeed, in the present study, limited RT-PCR studies identified the existence of a partial cDNA encoding for HERG in both SHSY5Y and HEK293 cells. Further sequencing would be required to determine if HEK293 cells contained the full sequence previously identified in SHSY5Y cells (Bianchi et al., 1998). In addition, rat cardiac myocytes, which are like HEK293 and CHO-K1 cells in being devoid of I_{Kr}, have an affinity for dofetilide of 290 nM (Duff et al., 1995). This value lies between the high affinity site for dofetilide (28 nM) in guinea pig myocytes (putatively I_{Kr}) and the low affinity site (1.63 μM) that supposedly does not reflect binding to I_{Kr}. Also, in a recent study the same authors (Geonzon et al., 1998) demonstrate high affinity binding for [^{3}H]dofetilide in human leukocytes. Interestingly, there is no evidence for a low affinity site in either neutrophils or mononuclear cells and the pharmacology of inhibition of [^{3}H]dofetilide binding is different to that of guinea pig myocytes. The IC_{50} values for dofetilide, E4031, clofilium and sotalol in human leukocytes are, however, similar to the data in the present study. As SHSY5Y and HEK293 are human cell lines, it may be prudent to consider that any alteration in pharmacology may reflect species differences. Geonzon et al. (1998) indeed postulate by a process of elimination that [^{3}H]dofetilide may bind to SK_{Ca} channels in leukocytes. In agreement with these authors, apamin (10 μM) did not inhibit [^{3}H]dofetilide binding in any of the cell lines examined in this paper; however, dofetilide (10 μM) did inhibit [^{125}I]apamin binding to rSK2 and rSK3 transfected cells by approximately 10% (Finlayson, unpublished observations). These findings appear to rule out a role for apamin-sensitive SK_{Ca} channels; however, that dofetilide inhibits or somehow interacts with the apamin-insensitive SK_{Ca} channel (Kohler et al., 1996) cannot be ruled out, as McLarnon and Wang (1991) demonstrated dofetilide inhibition of SK_{Ca}-mediated currents in CA1 hippocampal neurons with an IC_{50} of 400 nM. These cells express apamin-insensitive SK channels (Stocker et al., 1999).

The identification of a low affinity site for dofetilide and E4031 in the hamster cell line CHO-K1 is in good agreement with previously published data, as is the binding site density (Fiset et al., 1996). However, again there is very little electrophysiological evidence for the existence of K^+ currents (Robertson and Owen, 1993). Whether this data represents [^{3}H]dofetilide binding to a different site, a

different (non-functional) conformation of the same channel or as discussed above, species differences in the pharmacology of [^{3}H]dofetilide binding require further clarification (Ficker et al., 1998; Lees-Miller et al., 2000). Indeed, the lack of potassium channel currents in CHO-K1 cells and the inhibition of the stably expressed mouse Kv1.1 potassium channel current MK-1 by another class III antiarrythmic tedisamil, again highlights the potential for non-specific effects when using this class of compounds (Robertson and Owen, 1993).

Interestingly, the class III antiarrythmic WAY 123,398 had no effect on [^{3}H]dofetilide binding in any of the three cell lines examined, despite being able to functionally block HERG in SHSY5Y cells (Fig. 2c). Blockade of HERG by WAY 123,398 is voltage independent unlike that of E4031 and dofetilide (Faravelli et al., 1996) and WAY 123,398 works faster than dofetilide when applied extracellularly in SHSY5Y cells (Fig. 2c). These data provide some evidence for the hypothesis that WAY 123,398 has a different mechanism/site of action to other class III antiarrythmics; however, further studies are required to support this idea.

In conclusion, we have established conditions whereby we can examine the pharmacology of endogenous [^{3}H]dofetilide binding sites in the human cell lines SHSY5Y and HEK293. The pharmacological characteristics of the binding are similar in both cell lines despite no functional evidence for a HERG-like K^+ current in the latter. These findings cast doubt over whether [^{3}H]dofetilide binding in these cells could be used as a preclinical screen for I_{Kr} blockers. In addition, that [^{3}H]dofetilide is a ligand specific for I_{Kr}/HERG may need to be assumed with caution until the binding site detected in HEK293 cells can truly be identified.

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References

Arcangeli, A., Bianchi, L., Becchetti, A., Faravelli, L., Coronnello, M., Mini, E., Olivotto, M., Wanke, E., 1995. A novel inward-rectifying K^+ current with a cell-cycle dependence governs the resting potential of mammalian neuroblastoma cells. J. Physiol. 489 (2), 455–471.

Arcangeli, A., Rosati, B., Cherubini, A., Crociani, O., Fontana, L., Passani, B., Wanke, E., Olivotto, M., 1998. Long term exposure to retinoic acid induces the expression of IRK1 channels in HERG channel-endowed neuroblastoma cells. Biochem. Biophys. Res. Commun. 244, 706–711.

Bianchi, L., Wible, B., Arcangeli, A., Taglialatela, M., Morra, F., Castaldo, P., Crociani, O., Rosati, B., Faravelli, L., Olivotto, M., Wanke, E., 1998. HERG encodes a K^+ current highly conserved in tumours of different histogenesis: a selective advantage for cancer cells? Cancer Res. 58, 815–822.

Chadwick, C.C., Ezrin, A.M., O'Connor, B., Volberg, W.A., Smith, D.I., Wedge, K.J., Hill, R.J., Briggs, G.M., Pagani, E.D., Silver, P.J., Krafte, D.S., 1993. Identification of a specific radioligand for the cardiac rapidly activating delayed rectifier K^+ channel. Circ. Res. 72, 707–714.

Cheng, Y.C., Prusoff, W.H., 1973. The relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Chiesa, N., Rosati, B., Arcangeli, A., Olivotto, M., Wanke, E., 1997. A novel role for HERG K^+ channels: spike-frequency adaptation. J. Physiol. 501 (2), 313–318.

Duff, H.J., Feng, Z-P., Sheldon, R.S., 1995. High- and low-affinity sites for [^{3}H]dofetilide binding to guinea pig myocytes. Circ. Res. 77, 718–725.

Duff, H.J., Feng, Z-P., Fiset, C., Wang, L., Less-Miller, J., Sheldon, R.S., 1997a. [^{3}H]Dofetilide binding to cardiac myocytes: modulation by extracellular potassium. J. Mol. Cell Cardiol. 29, 183–191.

Duff, H.J., Feng, Z-P., Wang, L., Sheldon, R.S., 1997b. Regulation of expression of the [^{3}H]-dofetilide binding site associated with the delayed rectifier K^+ channel by dexamethasone in neonatal mouse ventricle. J. Mol. Cell Cardiol. 29, 1959–1965.

Faravelli, L., Arcangeli, A., Olivotto, M., Wanke, E., 1996. A HERG-like K^+ channel in rat F-11 DRG cell line: pharmacological identification and biophysical characterisation. J. Physiol. 496 (1), 13–23.

Ficker, E., Jarolimek, W., Kiehn, J., Baumann, A., Brown, A.M., 1998. Molecular determinants of dofetilide block of HERG K^+ channels. Circ. Res. 82, 386–395.

Finlayson, K., Butcher, S.P., Sharkey, J.S., Olverman, H.J., 1997. Detection of adenosine receptor antagonists in rat brain using a modified radioreceptor assay. J. Neurosci. Methods 77, 135–142.

Fiset, C., Feng, Z-P., Wang, L., Sheldon, R.S., Duff, H.J., 1996. [^{3}H]Dofetilide binding: biological models that manifest solely the high or the low affinity binding site. J. Mol. Cell Cardiol. 28, 1085–1096.

Geonzon, R., Exner, D.V., Woodman, R.C., Wang, L., Feng, Z-P., Duff, H.J., 1998. A high affinity binding site for [^{3}H]-dofetilide on human leukocytes. J. Mol. Cell Cardiol. 30, 1691–1701.

Jan, L.Y., Jan, Y.N., 1997. Cloned potassium channels from eukaryotes and prokaryotes. Annu. Rev. Neurosci. 20, 91–123.

Kiehn, J., Wible, B., Taglialatela, M., Brown, A.M., 1995. Cloned human inward rectifier K^+ channel as a target for class III methanesulfonanilides. Circ. Res. 77, 1151–1155.

Kiehn, J., Lacerda, A.E., Wible, B.A., Brown, A.M., 1996. Molecular physiology and pharmacology of HERG. Circulation 94, 2572–2579.

Kohler, M., Hirschberg, B., Bond, C.T., Kinzie, J.M., Marrion, N.V., Maylie, J., Adelman, J.P., 1996. Small-conductance calcium-activated potassium channels from mammalian brain. Science 273, 1709–1714.

Lees-Miller, J.P., Duan, Y., Teng, G.Q., Duff, H.J., 2000. Molecular determinant of high-affinity dofetilide binding to HERG1 expressed in *Xenopus* oocytes: involvement of S6 sites. Mol. Pharmacol. 57, 367–374.

Lynch, J.J., Baskin, E.P., Nutt, E.M., Guinosso, P.J., Hamill, T., Salata, J.J., Woods, C.M., 1995. Comparison of binding to rapidly activating delayed rectifier K^+ channel, I_{Kr}, and effects on myocardial refractoriness for classIII antiarrythmic agents. J. Cardiovasc. Pharmacol. 25, 336–340.

McLarnon, J.G., Wang, X-P., 1991. Actions of cardiac drugs on a calcium-dependent potassium channel in hippocampal neurons. Mol. Pharmacol. 39, 540–546.

Meyer, R., Heinemann, S.H., 1998. Characterisation of an eag-like

potassium channel in human neuroblastoma cells. J. Physiol. 508 (1), 49–56.

Mori, K., Hara, Y., Saito, T., Masuda, Y., Nakaya, H., 1995. Anticholinergic effects of class III antiarrythmic drugs in guinea pig atrial cells: different molecular mechanisms. Circulation 91, 2834–2843.

Pennington, A.J., Kelly, J.S., Finlayson, K., 2000. [^{3}H]Dofetilide binding in the presence and absence of recordable HERG-like potassium current in the human cell lines SHSY5Y and HEK293. J. Physiol. 523, 159.

Robertson, B., Owen, D.G., 1993. Pharmacology of a cloned potassium channel from mouse brain (MK-1) expressed in CHO cells: effects of blockers and an inactivation peptide. Br. J. Pharmacol. 109, 725–735.

Rosati, B., Rocchetti, M., Zaza, A., Wanke, E., 1998. Sulfonylureas blockade of neural and cardiac HERG channels. FEBS Lett. 440, 125–130.

Sanguinetti, M.C., Spector, P.S., 1997. Potassium channelopathies. Neuropharmacology 36 (6), 755–762.

Schonherr, R., Rosati, B., Hehl, S., Rao, V.G., Arcangeli, A., Olivotto, M., Heinemann, S.H., Wanke, E., 1999. Functional role of the slow activation property of ERG K^+ channels. Eur. J. Neurosci. 11, 753–760.

Snyders, D.J., Chaudhary, A., 1996. High affinity open channel block by dofetilide of HERG expressed in a human cell line. Mol. Pharmacol. 49, 949–955.

Spector, P.S., Curran, M.E., Keating, M.T., Sanguinetti, M.C., 1996. Class III antiarrythmic drugs block HERG, a human delayed rectifier K^+ channel: open-channel block by methanesulfonanilides. Circ. Res. 78, 499–503.

Stocker, M., Krause, M., Pedarzani, P., 1999. An apamin-sensitive Ca^{2+}-activated K^+ current in hippocampal pyramidal neurons. Proc. Natl. Acad. Sci. 96, 4662–4667.

Wang, H., Shi, H., Wang, Z., 1999. Inactivation block of the HERG human cardiac K^+ channels by RP58866. Br. J. Pharm. 127, 1899–1907.

Warmke, J.W., Ganetzky, B., 1994. A family of potassium channel genes related to *eag* in *drosophila* and mammals. Proc. Natl. Acad. Sci. 91, 3438–3442.

Wymore, R.S., Gintant, G.A., Wymore, R.T., Dixon, J.E., McKinnon, D., Cohen, I.S., 1997. Tissue and species distribution of mRNA for the I_{Kr}-like K^+ channel, *erg*. Circ. Res. 80, 261–268.

Zhou, Z., Gong, Q., Ye, B., Fan, Z., Makielski, C., Robertson, G., January, C.T., 1998. Properties of HERG stably expressed in HEK293 cells studied at physiological temperature. Biophys. J. 74, 230–241.

Zou, A., Curran, M.E., Keating, M.T., Sanguinetti, M.C., 1997. Single HERG delayed rectifier K^+ channels expressed in *Xenopus* oocytes. Am. J. Physiol. 272, H1309–H1314.